

Analysis of agouti signaling protein (*ASIP*) gene polymorphisms and association with coat color in Tibetan sheep (*Ovis aries*)

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ABSTRACT. Tibetan sheep, an indigenous breed, have a wide variety of phenotypes and a colorful coat, which make this breed an interesting model for evaluating the effects of coat-color gene mutations on this phenotypic trait. The agouti signaling protein (*ASIP*) gene is a positional candidate gene, as was inferred based on previous study. In our research, *ASIP* gene copy numbers in genomic DNA were detected using a novel approach, and the exon 2 g.100-104 mutation and copy number variation (CNV) of *ASIP* were associated with coat color in 256 sheep collected from eight populations with different coat colors by high-resolution melting curve assay. We found that the relative copy numbers of *ASIP* ranged from one to eight in Tibetan sheep. All of the g.100-104 genotypes in the populations were in Hardy-Weinberg equilibrium, and there was no relationship between the g.100-104 genotype and coat color ($P > 0.05$). The single *ASIP* CNV allele was found to be almost entirely associated with solid-black coat color; however, not all solid-black sheep displayed the putative single *ASIP* CNV genotype. From our

study, we speculate that the *ASIP* CNV is under great selective pressure and the single *ASIP* CNV allows selection for black coat color in Tibetan sheep, but this does not explain all black phenotypes in Tibetan sheep.

Key words: *ASIP*; Polymorphisms; Copy number variation; Coat color

INTRODUCTION

In mammalian species, coat color is an important breed characteristic and production trait. Coat color is determined by the amount of pigmentation and the ratio of eumelanin (black/brown) and pheomelanin (yellow/red), which are produced by melanocytes (Ito and Wakamatsu, 2003). Tibetan sheep are traditionally reared for production and without any structured genetic selection plan; they often have a white coat, but the head and limbs are covered with variegated wool. Tibetan sheep with variegated wool account for 90% of the total population, while solid-white or solid-black sheep are scarce. Therefore, we considered that Tibetan sheep could be an interesting model for identifying coat-color candidate genes. Although there are over 300 genes identified with known roles in mammalian pigmentation, a few key genes have been identified as major regulators of pigment production in domestic animals (Rieder et al., 2001; Liu et al., 2009; Suzuki, 2013).

The agouti signaling protein gene (*ASIP*) has been shown to be associated with coat-color production in a variety of animals; previous studies mapped the coat color locus on chromosome 13 for cats (Eizirik et al., 2003), mice (Kuramoto et al., 2001), foxes (Vage et al., 1997), horse (Rieder et al., 2001), cattle (Girardot et al., 2005), pigs (Drogemuller et al., 2006), dogs (Kerns et al., 2004; Schmutz et al., 2007), sheep (Norris and Whan, 2008), and humans (Kwon et al., 1994). The *ASIP* gene encodes a signaling protein (ASIP) that can act as an antagonist of the alpha-melanocyte-stimulating hormone (α -MSH), preventing the binding of α -MSH to melanocortin 1 receptor, which induces a decrease in the levels of cyclic adenosine monophosphate (cAMP). This occurs through a cascade of reactions that inhibit the formation of eumelanin, which results in lighter coat color. In this way, *ASIP* inhibits the production of eumelanin and leads to production of pheomelanin (Aberdam et al., 1998; Virador et al., 2000).

α -MSH combines with ASIP to regulate the formation of eumelanin and pheomelanin in melanoma cells (Hida et al., 2009). Studies have shown that recessive mutations that either impair ASIP protein function or abrogate ASIP expression result in more darkly pigmented phenotypes. Conversely, dominant mutations arising from upregulated expression of ASIP result in lighter phenotypes in some sheep breeds (Gratten et al., 2010). The dominant white color is caused by deregulated expression of the agouti protein and results from a 190-kbp genomic duplication that places a functional ASIP-coding sequence under the control of a duplicated promoter from the neighboring itchy homolog E3 ubiquitin protein ligase locus (Norris and Whan, 2008). The recessive self-color pattern is caused by the absence of ASIP expression, presumably arising from a *cis*-regulatory mutation that inactivates the promoter (Norris and Whan, 2008; Royo et al., 2008). ASIP expression was not detected in the skin tissue of the recessive black sheep with single copy *ASIP* alleles (Norris and Whan, 2008), and recessive self-color patterns may also result from the expression of functional mutations in exon 2 (Smit et al., 2002; Royo et al., 2008) and exon 4 (Norris and Whan, 2008). The contribution of *ASIP* to coat color patterns of domestic sheep has been confirmed to involve multiple alleles. Herein, we examine the mutation and copy number variation (CNV) of *ASIP* associated with coat color in Tibetan sheep.

MATERIAL AND METHODS

Animal and sample collection

Blood samples were taken from 256 Tibetan sheep belonging to eight local populations in northwest China, most of which were female. The coat color patterns expressed included solid-black, solid-white, and variegated. The eight populations were Guide Black Tibetan sheep and Minxian Black Tibetan sheep, which are two populations of sheep that are covered with black wool (Figure 1A); breeding Qinghai Tianjun White Tibetan sheep and Qilian White Tibetan sheep, the wool of which is white and sometimes the eye socket has small black spots/regions (Figure 1B); and Qinghai Ola Tibetan sheep, Gansu Ola Tibetan sheep, Langkazi Tibetan sheep, and Gansu Ganjia Tibetan sheep, which have white wool and black/brown spots on their body, and black/brown is usually present on the head and legs (Figure 1C). All eight populations live high in the mountains in harsh climatic conditions.

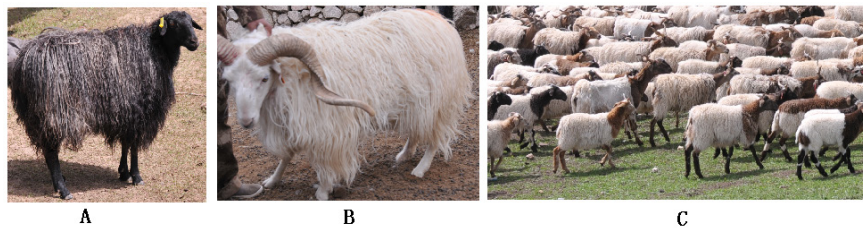


Figure 1. Coat color variation of Tibetan sheep: **A.** solid black, **B.** solid white, and **C.** variegated.

DNA extraction, amplification, and sequencing of the agouti locus

Genomic DNA was extracted from the collected blood samples using a TIANGEN DNA Mini Kit (TIANGEN Bio, China). A NanoDrop 2000 (Thermo Scientific, USA) was used to quantify the concentration of genomic DNA. Eight pairs of primers, ASIP 1-ASIP 8, were designed to characterize the sheep *ASIP* gene (Table 1), and the reference sequence

Table 1. Sequences of PCR primers.

Primer name	Primer sequence (5' to 3')	Products (bp)	Temperature (°C)
<i>ASIP</i> 1	CATTACTGGGGACCTATCAAC AGACAGAAGGGAAATCCAAC	504	55
<i>ASIP</i> 2	TGCTTCCTCAGTGCCTACAG CTCTTTCTTCTTTTCCGCTTC	1480	57
<i>ASIP</i> 3	AGTTCACATTGTCGGGATG GTTTGATGTCAATATATCCTTAG	1080	57
<i>ASIP</i> 4	GTTGCTTGCCACAAGTTCTA CTCCGCCATCAACACTTAAC	2320	59
<i>ASIP</i> 5	GACTCCGAACCTACCGTGAA CGCCCCAACGTCAATAGC	601	61
<i>ASIP</i> 6	CATTACTGGGGACCTATCAAC TATCGGCTTGGAGAGTGTGTTG	609	56
<i>ASIP</i> 7	CTGGCCTAAGTCCAAGATATG CCATTTCCTTCTCCAGTTTAGTTAG	440	55
<i>ASIP</i> 8	GGAAAGGGGAAGACAACAGAGAC ACTAGGCGAAGGGAAAAGAATG	668	56
Agt16	CAGCAATGAGGACGTGAGTTT		
Agt17	GTTTCTGCTGGACCTCTTGTTT	238	61
Agt18	GTGCCTTGTGAGGTAGAGATGGTGTT	242	60.5

(GenBank accession No. EU420023.1) was used to design primers for amplification of the 5353-bp coding region. Primers were synthesized by the Beijing Genomics Institution (BGI, China). Some of the PCR primers and conditions were previously reported (Norris and Whan, 2008). The mutations in the coding DNA sequence of *ASIP* were identified by sequencing. Mixed pools of DNA (Taylor et al., 1994) were obtained by mixing the DNA extracted from 10 black sheep and 10 white sheep. PCR products were sequenced by BGI, and single nucleotide polymorphisms were analyzed based on the sequencing peak diagrams.

Genotyping polymorphisms

The association between coat pattern and an *ASIP* functional mutation was quantified by genotyping the 5-bp deletion in 256 sheep. *ASIP* coding-region mutations (g.100-104delAGGA A in exon 2) were genotyped by designing primers near the genome location g.100-104: D5-F: 5'-TGAGGAAAGCCAGAGATG-3' and D5-R: 5'-CAGAAGGGAAATCCA ACAGG-3'. The PCR products were distinguished by the peaks of their melting curves. Then, three primers were used for detection of *ASIP* CNV by amplifying both the junction between the duplicated DNA copies and the 5'-breakpoint sequences (Norris and Whan, 2008) (Agt16, Agt17, and Agt18; Table 1). Primers Agt16 and Agt18 spanned a unique 242-bp PCR product, which includes the junction between the duplicated copies, while Agt16 and Agt17 can produce a 238-bp fragment that can cover the 5'-breakpoint sequence (Figure 2) according to the high-resolution melting curve assay (HRM) melting peaks of products that we can easily be used to detect the CNV.

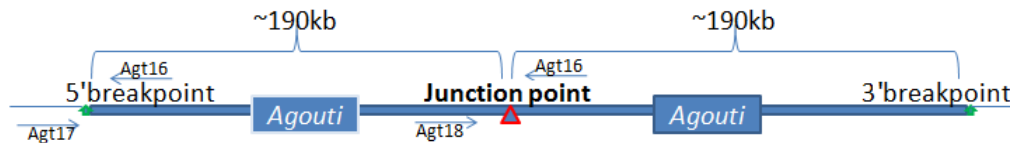


Figure 2. Structure of the *ASIP* gene duplication in genomic DNA.

ASIP CNV numbers assay

To estimate sheep *ASIP* CNV, we made a pMD19-agouti fragment clone plasmid using standard construction techniques. We chose a conserved fragment between intron 3 and exon 4 of *ASIP* using DNA and mRNA sequences (GenBank EU420023.1 and NM_001134303.1) as references to design a primer to amplify a 193-bp product: *ASIP*-F: 5'-GTTAAGTGTGATG GCGGAG-3' and *ASIP*-R: 5'-GTTCTTCATCGGAGCCTTTC-3'. PCR was performed in a 50- μ L reaction volume containing 50-100 ng DNA, LA Taq (5 U/ μ L) (Takara, China), 0.5 μ L 10X LA-PCR buffer, 5 μ L dNTP mixture, 8 μ L 10 μ M *ASIP*-F and *ASIP*-R per 2 μ L, with ddH₂O added to bring the solution to 50 μ L. PCR conditions were 4 min at 95°C, 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C for 40 cycles, and 5 min at 72°C (Veriti 96 well, Applied Biosystems, USA). The PCR product was subjected to 2% agarose gel electrophoresis, and the target band was recovered and purified using an agarose gel DNA extraction kit (TIANGel, Beijing, China); fragments were connected with a pMD19-T vector (TaKaRa, China). The spliced product was transformed into *E. coli* JM109-competent cells (TaKaRa, China) and coated onto agar plates that contained isopropyl- β -d-thiogalactoside, 5-bromo-4-chloro-3-indolyl

β -D-galactoside and ampicillin. The white-positive colonies were screened for culturing, and positive clone plasmids were sequenced for identification.

The plasmid concentration was measured using the NanoDrop 2000, and the copy number of plasmids per microliter was calculated using the following equation: $\text{copies}/\mu\text{L} = (6.02 \times 10^{23}) \times (\text{ng}/\mu\text{L} \times 10^{-9}) / (\text{DNA length} \times 660)$. The plasmid DNA length was 2885 bp, which was composed of the sum of the vector length and the PCR product. The standard plasmid was diluted to a concentration of 10^8 copies/ μL , and then it was diluted from 10^7 to 10^2 (copies/ μL) by six gradients. The standard curve was made using real-time quantitative PCR with five concentrations (10^2 - 10^6) as standards (three replicates per concentration). Real-time quantitative PCR was performed in an 18- μL reaction volume containing 9 μL 2X SYBR Premix Ex Taq TaKaRa, China), 0.4 μL Rox Reference Dye, 0.8 μL 10 μM upstream and downstream primers, 6 μL ddH₂O, and 1 μL standard plasmid DNA, and each sample was analyzed three times. Real-time quantitative PCR conditions were 3 min at 95°C, 10 s at 95°C, and 30 s at 60°C for 40 cycles (Bio-Rad CFX-96, USA). The mean Ct value of each sample was measured to calculate the copies according to the linear regression equation of the standard curve. Finally, the DNA from thirty sheep randomly selected was checked using this new approach.

Statistical analysis

The association between *ASIP* gene polymorphisms and coat-color phenotype was analyzed using the Fisher exact test (SHEsis; <http://analysis.bio-x.cn>), and the Hardy-Weinberg equilibrium of genotype frequencies was detected by χ^2 test in SAS version 9.2 (SAS Institute Inc., USA).

RESULTS

Mutations in Tibetan sheep *ASIP*

Thirty-five mutation sites were found in the full-length sequence from exon 2 to the 3'UTR of *ASIP*, including three mutation sites in the exon region (g.100-104 deletion; g.5051G>C; g.5172T>A) and 32 in the intron region (Table S1). We focused our study on a 5-bp deletion in exon 2 (g.100-104delAGGAA, denoted as D5). The D5 deletion would result in a frame shift followed by a premature stop codon that is 63 amino acids downstream of the start site, truncating the agouti protein before the functionally important cysteine signaling domain (amino acids 91-130) (Norris and Whan, 2008), while N5 does not have the AGGAA deletion. HRM was used to genotype the g.100-104 site based on melting peaks, and we only found two genotypes: D5N5 and N5N5 (Figure 3). The result

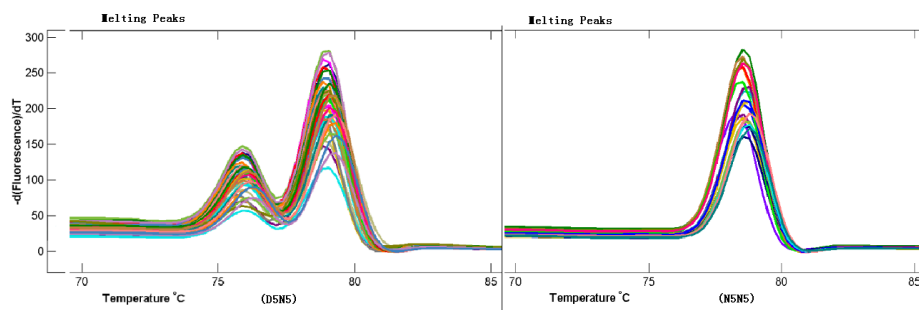


Figure 3. High-resolution melting curve assay genotyping.

of the sequencing was consistent with HRM genotype, which just have two genotypes (Figure 4). We estimated the frequency of the D5N5 and N5N5 alleles in the 256 sheep by applying the Hardy-Weinberg equilibrium. The most frequent allele was N5N5, and D5N5 was less frequent (Table 2). We compared pairwise population measures of the three coat patterns for both types of coat color. χ^2 analysis showed that both g.100-104 genotypes were in Hardy-Weinberg equilibrium. There was no relationship between the g.100-104 genotype and coat color, as determined by a comparative analysis of correlation (Fisher's exact test, $P > 0.05$).

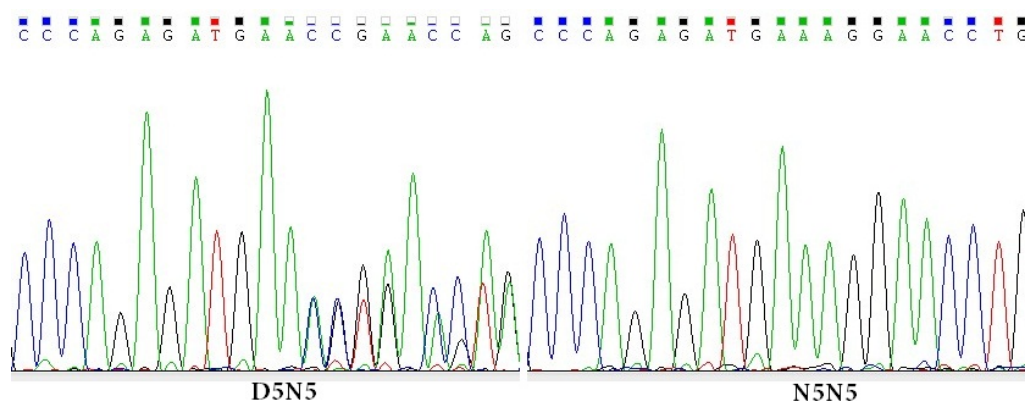


Figure 4. DNA sequencing of the *ASIP* gene g.100-104.

Table 2. Genotypes of the *ASIP* exon 2 g.100-104 deletions and the *ASIP* CNV.

Coat color	Local strains	Multiple CNVs		Single CNV	
		N5D5	N5N5	N5D5	N5N5
Solid white	QLW (40)	18	21	-	1
	TJW (24)	8	11	2	3
Solid black	GDB (40)	4	18	3	15
	MXB (56)	20	10	10	16
Variegated	GSOL (27)	15	12	-	-
	QHOL (21)	9	12	-	-
	GJ (24)	12	11	-	1
	LKZ (24)	1	23	-	-

QLW = Qilian White Tibetan sheep; TJW = Qinghai Tianjun White Tibetan sheep; GDB = Guide Black Tibetan sheep; MXB = Minxian Black Tibetan sheep; GSOL = Gansu Ola Tibetan sheep; QHOL = Qinghai Ola Tibetan sheep; GJ = Gansu Ganjia Tibetan sheep; LKZ = Langkazi Tibetan sheep.

ASIP CNV analysis

The gradients of 10^2 - 10^6 copies/ μ L plasmid DNA were amplified by real-time quantitative PCR according to the values of Ct and log copies. The program drafted a standard curve ($R^2 = 0.999$, $E = 104.6\%$), the linear equation $y = -3.215\log x + 35.8$ (Figure 5). The amplification efficiency of genomic DNA was $E = 98\%$, indicating that the standard curve could be used to detect gene copy numbers of *ASIP*.

Only two of the thirty sheep with a single copy allele containing the *ASIP* were detected and were named A1 and A2. We used the *ASIP* copy number of A1 as a control sample

to calculate the relative copy number of other samples. The relative copy number of A2 was 0.98; the relative copy numbers of other individuals ranged from two to eight ([Table S2](#)).

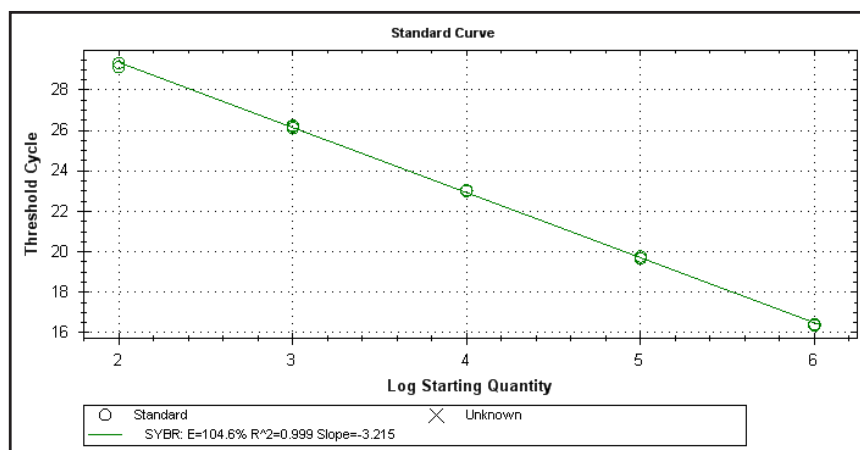


Figure 5. Standard and dynamic curves by real-time quantitative PCR.

Association between coat pattern and *ASIP* CNV

We analyzed the *ASIP* CNV of 256 Tibetan sheep belonging to eight local populations with the HRM assay, which is designed to test the presence of a duplicated copy allele with single or multiple *ASIP* CNVs. According to the different dissolution peaks for genotyping using LightScanner (Idaho Technology, Inc., USA), there were three types of dissolution peaks, which means they were amplified differently ([Figure S1](#)). Only one of the 96 variegated sheep and six of the 64 solid-white sheep had a single *ASIP* CNV allele, while 44 of the 96 solid-black sheep had a single *ASIP* CNV allele (Table 2). The association between the single *ASIP* CNV allele and mostly dark coat color (when all sheep were analyzed together) was highly significant (Fisher's exact test; solid black vs solid white, $P < 0.01$; solid black vs variegated, $P < 0.01$). We found a departure of the CNV allele from Hardy-Weinberg equilibrium in both pairwise-compared populations (χ^2 test; solid black vs solid white, $P < 0.01$; solid black vs variegated, $P < 0.01$), indicating that *ASIP* CNV is under great selective pressure.

DISCUSSION

The coding region of the *ASIP* gene is determined by three exons (2, 3, and 4, according to the nomenclature in mice). The full-length (approximately 5353 bp) sequence from exon 2 to the 3'UTR of *ASIP* was amplified and sequenced in our study. After splicing and assembly, we discovered 35 mutation sites, all of which have been previously reported in domestic sheep (Norris and Whan, 2008). In sheep, the ASIP protein consists of 133 amino acids that comprise a signal peptide and functional amino acids. Previous studies showed *ASIP* mutations related to animal coat color but mainly addressed the *ASIP* functional-deletion mutation and missense mutations. *ASIP* alleles causing coat-color variation have also been characterized in foxes, in which exon 2 is completely missing (Vage et al., 1997); rats, which

have a 19-bp deletion within the coding DNA sequence (Kuramoto et al., 2001); cats, which have a 2-bp deletion in exon 2 (Eizirik et al., 2003); horses, which have an 11-bp deletion in exon 3 (Rieder et al., 2001); dogs, which have a missense mutation in exon 4 (Schmutz et al., 2007); and sheep, which have a 5-bp deletion in exon 2 and a missense mutation in exon 4 (Norris and Whan, 2008). The sheep *ASIP* exon 2 g.100-104 AGGAA deletion is a frameshift mutation that causes termination of the amino acids coding at locus 64 and results in the lack of a functional mature polypeptide signal domain (Royo et al., 2008; Gratten et al., 2010). Additionally, Gratten et al. (2010) demonstrated that the regulatory exons of *ASIP* are important in controlling expression in different breeds of sheep. However, that study revealed no relationship between g.100-104 genotypes and coat color, and the D5D5 genotype was not detected in that study.

The linear equations constructed using a standard curve to calculate the copy number of *ASIP* in unknown DNA showed a range of 10^6 - 10^2 copies with a linear relationship ($R = 0.999$) and amplification efficiency $E = 104.6\%$, indicating that the standard curve had high accuracy. The results obtained using this method to determine single or multiple copies of *ASIP* are consistent with the results reported by Norris and Whan (2008). Compared with the microarray, this method for establishing *ASIP* CNV using SYBR Green real-time quantitative PCR is simple, fast, and economical. Previous data from Norris and Whan (2008) on asymmetric competitive PCR copy-number assays of recessive black and white Merino sheep revealed that recessive black Merino sheep always have a single *ASIP* copy, while white Merino sheep can have two to five *ASIP* copy alleles. Our calculations of relative copy numbers of *ASIP* range from one to eight in Tibetan sheep. Calculation of the *ASIP* copy numbers using this method was consistent with the findings of Norris and Whan (2008); in comparison with the method they designed, our approach can be used to easily detect CNVs in a typical lab like ours.

In sheep, *ASIP* is characterized by CNV, which determines the white and tan (A^{Wt}) agouti allele and white coat color, whereas non-agouti black sheep with a single copy of *ASIP* could carry a regulatory mutation in an unidentified regulatory region, deletions in exon 2, or a missense mutation in exon 4 (Norris and Whan, 2008; Royo et al., 2008; Gratten et al., 2010). Association between coat color and *ASIP* CNVs was highly significant in the populations of solid-black vs solid-white or variegated sheep; our study found that the single *ASIP* CNV allele was almost entirely associated with dark coat color. These results confirmed that the *ASIP* locus affects coat color in sheep.

We also found that Tibetan sheep with the three coat-color patterns had both single and multiple *ASIP* CNV alleles. Norris and Whan (2008) found that all-white Merinos had at least one duplicated *ASIP* allele, while all of the recessive black Merinos contained only a single allele copy. This was indirectly confirmed by Fontanesi et al. (2011), who showed that a copy of a duplicated allele produces grey coat color in Massese sheep. It was predicted that at least one duplication of the genomic region, including a functional copy of the *ASIP*, is necessary to produce the white phenotype. Our research revealed that one of the 96 variegated sheep and six of the 64 solid-white sheep had a single *ASIP* CNV allele. Interestingly, another study recently identified a copy-number variant in goat *ASIP*, where animals carrying a duplicated *ASIP* allele were not completely associated with white coat color, and black coat color in goats was not completely associated with any other identified missense mutation of this gene (Fontanesi et al., 2010). *ASIP* CNV is under great selective pressure, and it can be speculated that the single copy of the *ASIP* is more associated with Tibetan sheep with black coat color.

Thus, we conclude that there are other major pigment genes responsible for the presence of pigmented fibers in Tibetan sheep.

A genome-wide association study (GWAS) of most of the world's sheep breeds revealed that the *KIT*, *ASIP*, and *MITF* genes that determine the sheep coat color have been under strong selection (Kijas et al., 2012). However, *ASIP*, which controls a series of alleles of black and white coat color, was not detected in a new GWAS of sheep selection (Zhang et al., 2013), although they used a different sheep breed. Tibetan sheep live in unique geographic environment that causes natural selection in breed internal, indicating no artificial selection for the phenotypes of coat color. Unlike domestic sheep such as Merino (Norris and Whan, 2008), Dubian, Privorian (Fontanesi et al., 2012), and Massese sheep (Fontanesi et al., 2011), which are likely to have been influenced by strong artificial selection for various wool colors, the wool color of Tibetan sheep is not the main selection trait. *ASIP* is the major regulatory gene for coat color of modern Western sheep, primitive feral-breed Soay sheep (Gratten et al., 2010), and the ancient caprinae species of Barbary sheep (Norris and Whan., 2008). In our study, we revealed that Tibetan sheep may also be affected by *ASIP*, and then presumably there may be other genes in addition to *ASIP* involved in coat-color regulation.

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[Supplementary material](#)

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